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Extension of *in vivo* half-life of biologically active molecules by XTEN protein polymers

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ABSTRACT

XTEN™ is a class of unstructured hydrophilic, biodegradable protein polymers designed to increase the half-lives of therapeutic peptides and proteins. XTEN polymers and XTEN fusion proteins are typically expressed in *Escherichia coli* and purified by conventional protein chromatography as monodisperse polypeptides of exact length and sequence. Unstructured XTEN polypeptides have hydrodynamic volumes significantly larger than typical globular proteins of similar mass, thus imparting a bulking effect to the therapeutic payloads attached to them. Since their invention, XTEN polypeptides have been utilized to extend the half-lives of a variety of peptide- and protein-based therapeutics. Multiple clinical and preclinical studies and related drug discovery and development efforts are in progress. This review details the most current understanding of physicochemical properties and biological behavior of XTEN and XTENylated molecules. Additionally, the development path and status of several advanced drug discovery and development efforts are highlighted.

1. Introduction

Over the past 30 years the biopharmaceutical industry has developed many pharmaceuticals based on physiologically active peptides and proteins such as hormones, enzymes, cytokines, antibodies and antibody fragments, and growth and coagulation factors. As natural components of physiological pathways, biological therapeutics tend to be highly selective, efficacious and safe. A number of therapeutic peptides and proteins are administered as replacement therapy to patients with clinical manifestation of deficiency, e.g. diabetes, hemophilia and growth hormone deficiency (GHD). Biologics come with some limitations, however. First, with very few exceptions, they cannot be given orally but must be administered by injection. Second, peptides and most proteins have characteristically short half-lives in circulation and are often cleared from the body within hours, if not minutes, thus necessitating frequent injections during the course of treatment. Thus, to facilitate the acceptance of new protein therapeutics and improve patient compliance, significant efforts have been directed toward development of various half-life extension technologies.

The strategies to extend half-lives of therapeutic proteins are diverse, with most approaches either linking the drug to bulking moieties, or enabling FcRn-mediated recycling. Bulking approaches aim to slow down renal clearance by increasing the hydrodynamic volume of the biologically active molecule. In addition, bulking moieties may shield the molecule from proteolytic degradation and detection by the host immune system, and decrease clearance receptor binding. Polyethylene glycol (PEG), historically the first bulking agent commonly employed to improve *in vivo* half-life of biotherapeutics, has been incorporated into multiple approved products (as reviewed in [1–4]). However, success in developing PEGylated pharmaceuticals has been accompanied by rising concerns. PEG is not biodegradable, and cellular vacuolation has been observed for PEGylated compounds in animal studies [5–7]. Despite a general consensus that PEG itself is not immunogenic, immunogenicity of PEGylated therapeutics was reported and appeared to be dependent on payload, PEG size and chemical composition. The specificity and neutralizing properties of anti-PEG antibodies detected in patients treated with PEGylated proteins remain controversial [8–10]. Occurrence of anti-PEG antibodies in healthy population has also been reported; and hypothesized to result from exposure to PEG and PEG-containing compounds in cosmetics, pharmaceuticals and processed foods [11–13]. The biological impact of anti-PEG antibody expression in this population is unclear. Both the success and limitations of PEG have spurred development of alternative biodegradable bulking agents, such as natural and semi-synthetic polysaccharides, including *O*- and *N*-linked oligosaccharides, dextran, hydroxyethyl starch (HES), polysialic acid and hyaluronic acid [14,15], as well as unstructured protein polymers such as homo-amino acid polymers, elastin-like polypeptides, XTEN and PAS [14,16].

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The second half-life extension approach exploits recycling through the FcRn receptor (reviewed in [14,17,18]). Two plasma proteins, serum albumin and IgG, have exceptionally long half-lives in circulation. When taken up by cells through nonspecific endocytosis, both proteins bind to the FcRn receptor in acidic endosomes and become protected from intracellular degradation. The protein-receptor complexes are recycled to the cell surface, where albumin and immunoglobulin molecules are released back into circulation at the neutral pH of the plasma. Several methods have been used to exploit the increased half-life of IgG or albumin for therapeutic payloads: (1) genetic fusion of the payload to the Fc domains of IgG or to the albumin polypeptide; (2) chemical conjugation of the payload to the albumin, IgG or Fc domain; (3) fusion or conjugation to mediator molecules which physically bind to IgG or albumin. Examples of such mediators are IgG-binding peptides, fatty acids, albumin-binding peptides, and albumin-specific antibody fragments [14,16].

This review focuses on XTEN, a class of unstructured biodegradable protein polymers developed by Amunix to increase the half-lives of therapeutic peptides and proteins genetically fused or chemically conjugated to them. XTEN polymers were conceived as non-immunogenic polypeptides consisting of the six hydrophilic, chemically stable amino acids A, E, G, P, S and T. In order to create genetically and chemically stable polypeptides that lack secondary structure and that are highly expressed in *Escherichia coli*, a library of non-repetitive 36 amino acid-long segments was generated, and nearly 1500 unique segments were screened for expression and biophysical properties. Selected segments were iteratively ligated, and the resulting molecules were rescreened for maximal expression, yielding a series of 864 residue-long polypeptides. The five most highly expressed of these polypeptides were tested for genetic stability, solubility in aqueous media, heat stability, and propensity to aggregate. Emerging from the selection process was the polypeptide designated as XTEN [19]. The prototype molecule was followed by development and characterization of multiple derivatives based on the parental XTEN molecule, and today, the name XTEN applies to the entire group of polypeptides. The physicochemical and biological properties of XTEN protein polymers, their manufacturability and specific applications are discussed below.

2. XTEN protein polymers

2.1. Physicochemical properties and analytics of XTEN protein polymers

The first 864 amino acid residue XTEN (XTEN864) polypeptide composed of A, G, E, P, S, and T was described in 2009 [19]. The selected sequence formed the basis for the physicochemical properties displayed by XTEN, including lack of a secondary structure, very high solubility and low propensity toward aggregation. In the following years, shorter XTEN polypeptides representing fragments of the parental 864 amino acid-long molecule were explored [20,21]. While standard cloning techniques theoretically could permit any number of amino acid residues to be incorporated in the XTEN polypeptide, to aid in practical and systematic evaluation, additional XTEN polymers were produced and characterized at defined fractional sizes, including 2/3 of 864 (576 residues), 1/2 (432 residues), 1/3 (288 residues), and 1/6 (144 residues). This section describes common protein analytical methods applicable to XTEN protein polymers and highlights key differences between XTEN and regular globular proteins and non-protein polymers. The analytical methods we used to characterize XTEN include size-exclusion chromatography (SEC), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electrospray ionization mass spectrometry (ESI-MS), reversed-phase high-performance liquid chromatography (RP-HPLC), hydrophobic interaction chromatography (HIC), anion exchange chromatography (AEX), dynamic light scattering (DLS), and viscometry.

Size-exclusion chromatography (SEC) is a widely used technique to characterize macromolecules such as polymers and proteins [22,23]. Macromolecules are separated by SEC based on their hydrodynamic volumes, which in turn correlate with calculated molecular weights (MW) for globular proteins. For XTEN polypeptides lacking secondary structure, abnormally large hydrodynamic volumes were observed [19,20]. To explore this property further, XTEN polymers ranging from 144 to 1728 amino acid residues in length were mixed and analyzed using a size-exclusion column with 500 Å pore size beads. For comparison, a standard mixture of globular proteins was analyzed in parallel (Fig. 1, A, B). As a group, XTEN polymers characteristically elute earlier than

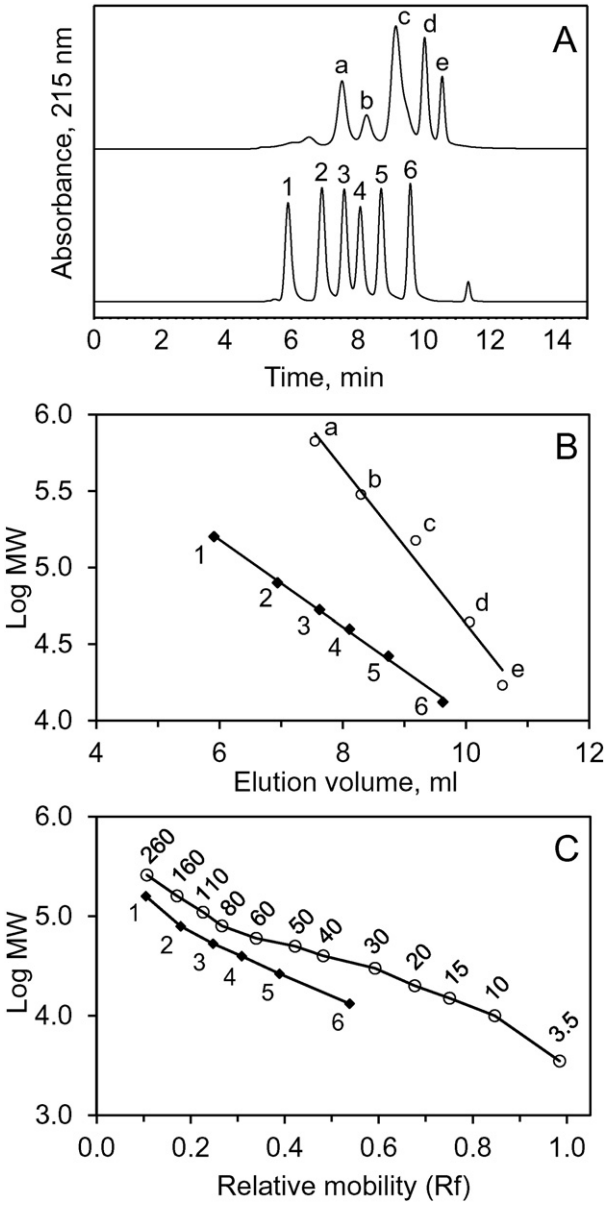

Fig. 1. SEC and SDS-PAGE characterization of XTEN polymers. XTEN864 (2), XTEN576 (3), XTEN432 (4), XTEN288 (5) and XTEN144 (6) were prepared as described in [21,24]. XTEN1728 (1) was prepared by cross-linking two XTEN864 molecules. The Phenomenex (Torrance, CA) column performance check standard mixture, prepared with bovine thyroglobulin (a), IgA (b), IgG (c), ovalbumin (d) and myoglobin (e), was used for comparison. Panel A: analytical SEC was performed using a Yarra™ 3 μm SEC-4000, 300 × 7.8 mm column (Phenomenex). The mobile phase consisted of 50 mM sodium phosphate, 300 mM sodium chloride, pH 6.5 buffer. Elution was performed at a flow rate of 1 mL/min. The upper chromatogram shows separation of globular proteins (column performance check standard), and the lower chromatogram shows the separation of XTEN polymers of different lengths. Panel B: comparison of molecular weight calibration curves for globular protein standards (open circles) with XTEN polymers of different length (filled squares). Panel C: migration distances for SDS-PAGE molecular weight markers (open circles, MW in kDa) compared with XTEN polymers of different length (filled squares).

globular proteins despite their significantly smaller size by MW. For example, the 53 kDa XTEN576 eluted at essentially the same elution time as the 670 kDa thyroglobulin, and the 79 kDa XTEN864 eluted as the equivalent of a 1.5 MDa globular protein. Elution times for XTEN polymers were inversely proportional to the logarithm of the MW values. Notably, within the group, XTEN polymers deviated significantly less from the regression line than the globular proteins. Thus, regardless of length, all XTEN polymers behaved as a homologous class of random coil polymers. This property of XTEN is essential for tuning the pharmacokinetic properties of therapeutic payloads in a predictable manner. Also, this property suggests that XTEN biodegradation upon administering an XTENylated drug will not result in a sudden loss of drug function, but rather will cause a gradual shortening of drug half-life. The relationship between XTEN length and the half-life is discussed in Section 3.1. While SEC is sensitive to the size of XTEN and thus can be used to characterize aggregates in protein preparations, adding a fusion protein or conjugation of peptide payload to XTEN864 showed no significant effect on its elution profile [24].

SDS-PAGE, the most commonly used technique for protein analysis, has been used for XTEN in applications ranging from testing XTEN expression in *E. coli* to characterization of highly purified final products. Coomassie Blue stain, generally used to visualize proteins in an SDS-PAGE gel, does not stain XTEN itself and can be used only for the analysis of fusions and conjugates [19–21,25]. XTEN polymers can be visualized on the gel with silver staining [24], though the method is not as sensitive as for regular proteins consisting of all 20 natural amino acids. The most suitable staining reagent for XTEN identified thus far is Stains-All, a cationic carbocyanine dye used to stain highly anionic proteins and nucleic acids [26,27]. Stains-All dye stains XTEN polymers as dark blue bands with very high sensitivity (see Biodegradation Section 3.2). By contrast, natural proteins typically stain yellow, red or pink. This differential staining property of Stains-All dye is advantageous in identifying XTEN polymers and their conjugates against a background of native proteins present in biological matrices. XTEN polymers migrate slower relative to natural proteins of similar molecular weight, likely due to their weak binding of SDS caused by the absence of hydrophobic amino acids. The 79 kDa XTEN864, for example, migrates similarly to a 160 kDa polypeptide marker [24]. Fig. 1, C illustrates protein mobility as function of XTEN length. Similar to SEC analysis, relative migration distances of individual XTEN polymers correlate logarithmically to MW with minimal deviation.

Intact MW analysis by high-resolution mass spectrometry (MS) is another useful tool for the chemical characterization of biotherapeutics. Analysis of PEGylated proteins with MS methods yields broad mass distribution spectra due to PEG polydispersity [28–31]. Several approaches for MS analysis of PEGylated proteins have been evaluated, but challenges persist due to the heterogeneity inherited from PEG [32]. By contrast, ESI-MS analysis of XTEN polymers yields single monodisperse peaks with experimental values matching calculated MWs [24]. This is a consequence of XTEN polymers being expressed as DNA-encoded proteins with precisely defined length and composition. This property clearly distinguishes XTEN from polydisperse synthetic polymers such as PEG and polysaccharides such as hydroxyethyl starch (HES). Fig. 2, A shows a panel of representative reconstructed zero-charge ESI mass spectra for 144–864 amino acids-long XTEN. Minor peaks are usually observed exactly at 1/2 and 1/3 of full-length MW. No corresponding bands have been detected by SDS-PAGE analysis of the same protein preparations. These minor peaks represent artifacts of Bayesian reconstruction and may be ignored. The monodispersity of XTEN allows the full advantage of MS as an analytical method. Even small changes to the protein composition, such as conjugation of a 4-(*N*-maleimidomethyl)cyclohexanecarboxamido group [24], or loss of a single N-terminal amino acid from the peptide fusion [25], have been detected and identified by analysis of mass difference.

RP-HPLC is a sensitive and quantitative technique for the analysis of small molecules and peptides, but its use for larger proteins is limited by

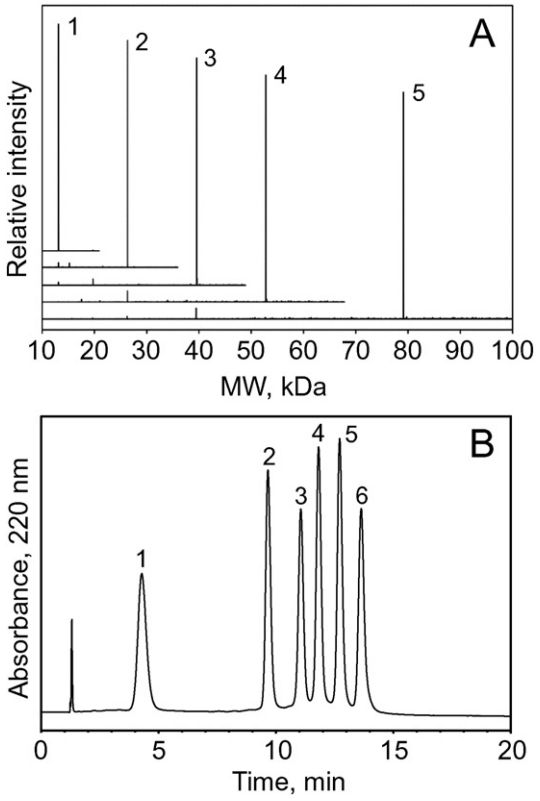

Fig. 2. Characterization of XTEN polymers (XTEN144 (1), XTEN288 (2), XTEN432 (3), XTEN576 (4), XTEN864 (5) and XTEN1728 (6)) by ESI-MS and HIC. Panel A: combined ESI-MS spectra. Analysis was performed by desalting the sample offline, then infusing into a calibrated QSTAR XL mass spectrometer (AB Sciex) [24]. Panel B: analytical HIC was performed using a 4.6 × 100 mm ProPac HIC-10 column (Thermo Scientific). Mobile phase A: 50 mM sodium phosphate, 1.5 M ammonium sulfate, pH 7.0 buffer. Mobile phase B: 50 mM sodium phosphate, pH 7.0 buffer. Gradient: 10–80% mobile phase B, 20 min at 1 mL/min.

the typically poor solubility of globular proteins in organic solvents. Hydrophilic unstructured XTEN polymers of all sizes have been found to be compatible with high concentrations of organic solvents, and therefore with RP-HPLC methods. XTEN polymers bind to C4–C18-wide pore (300 Å) matrices and quantitatively elute from the columns as sharp peaks with a full-width-at-half-maximum (FWHM), which is essentially the same as observed for short synthetic peptides [21,24]. Since XTEN polymers lack UV absorbance at 280 nm, protein quantitation is performed by RP-HPLC at 215–220 nm using reference standards established by quantitation via amino acid analysis (AAA). The hydrophilicity and monodispersity of XTEN render RP-HPLC to be a highly sensitive analytical tool to almost any modification in the chemical structure of the XTENylated molecules. The method has been used to monitor chemical conjugation reactions or detect post-translational modifications of genetically fused or conjugated payloads [24]. When analyzing unmodified XTEN, however, the elution profiles do not vary substantially with the length of the XTEN polymer.

RP-HPLC and MS analysis can also be combined with proteolytic degradation for detailed peptide mapping studies. These experiments are used for structural sequence confirmation and identification of post-translational modifications in proteins [33]. Trypsin, the most commonly used enzyme for peptide mapping, does not cleave XTEN polymers due to a lack of internal Lys and Arg residues. Conversely, XTEN does not interfere with efficient trypsin digestion of fused or conjugated payloads.

HIC, which is commonly used for the characterization of large proteins such as monoclonal antibodies (mAbs) and antibody drug

conjugates (ADCs) [34], has been found to be useful for characterization of XTEN polymers. In contrast to RP-HPLC, HIC efficiently separates XTEN polypeptides based on polymer length, with shorter XTEN chains eluting early in the salt gradient and longer ones eluting later (Fig. 2, B). The method has been found to be suitable as an analytical tool to monitor truncations of XTEN polymers in *E. coli* crude homogenates and during early purification steps. C-terminal truncations likely result from premature termination of polypeptide biosynthesis. They lack a C-terminal purification tag and thus can be efficiently removed by tag-selective chromatography. N-terminal truncations, likely occurring from unspecific proteolysis intracellularly or in crude extracts, can be removed by the final AEX polishing step [24]. Highly purified full-length XTEN polymers elute from HIC columns as symmetric peaks (Fig. 2, B) while the presence of truncated XTEN species can be detected as asymmetric ascending shoulders of the product peaks (data not shown).

AEX is useful for both purification and characterization of the anionic XTEN polymers and their conjugates. Similar to HIC, analytical AEX can also be used as a size-based method for the analysis of different length XTEN polymers and their conjugates (data not shown).

Aggregates of protein- and polymer-based therapeutics are known to be significant sources of drug immunogenicity and pose a significant efficacy and safety risk [35]. DLS is an informative tool that may be used to monitor aggregation in solutions [36]. DLS volume distribution analysis of XTEN864 polymer showed single peaks with low polydispersity index values at concentrations tested up to 100 mg/mL (Fig. 3, A), reflecting the intrinsic solubility-enhancing properties of the XTEN polymer. Further studies performed using DLS with XTEN polymers under various conditions suggest that XTEN polymers do not aggregate in physiologically compatible solutions. At elevated temperatures, which normally lead to irreversible denaturation and non-specific aggregation of proteins, aggregation of XTEN is not observed. Fig. 3, B shows a comparative analysis of human serum albumin and XTEN864 under increasing temperatures. While albumin exhibited progressive aggregation at temperatures above 65 °C, XTEN polymer demonstrated excellent thermal stability at temperatures as high as 90 °C.

Viscosity of a therapeutic protein solution is an important factor affecting the administration of the parenteral drug via syringe [37]. A solution viscosity below 50 cP is generally preferred for subcutaneous injection of therapeutic proteins [38]. The absolute viscosity of XTEN864 solution, concentrated to 100 mg/mL, was measured to be 26–30 cP, depending on the buffer system used. Depending on the attached payload, it is likely that XTEN864 conjugates up to this concentration are amenable for subcutaneous administration through a fine (27–31 gauge) needle.

Rheological measurements can also provide information regarding the structural conformation of a polymer. For a series of chemically related polymers, a logarithm of intrinsic viscosity correlates linearly to the logarithm of MW (Mark–Houwink–Kuhn–Sakurada equation; [39]); the slope of the trace denotes the polymer's structural conformation in solution, with 0 corresponding to compact sphere, 0.5–0.8 to random coil and 1.8 to rigid rod [39,40]. Intrinsic viscosities of different length XTEN polymers in PBS at 25 °C were measured and a double logarithmic plot of intrinsic viscosity vs MW prepared (Fig. 3, C). The slope of the simple regression line suggests that XTEN polymers behave like random coils at physiological pH [39]. Further studies with XTEN protein polymers and conjugates are in progress to learn their rheological properties.

Together these analytical techniques highlight the characteristics of XTEN that make it a useful bulking agent for half-life extension of therapeutic peptides and proteins. They also serve as predictive tools for the properties of XTENylated fusion products or XTEN-conjugated payloads to ensure that such molecules will have the desired physicochemical and clinical characteristics, such as long half-life in circulation, high solubility, and ease of manufacturing.

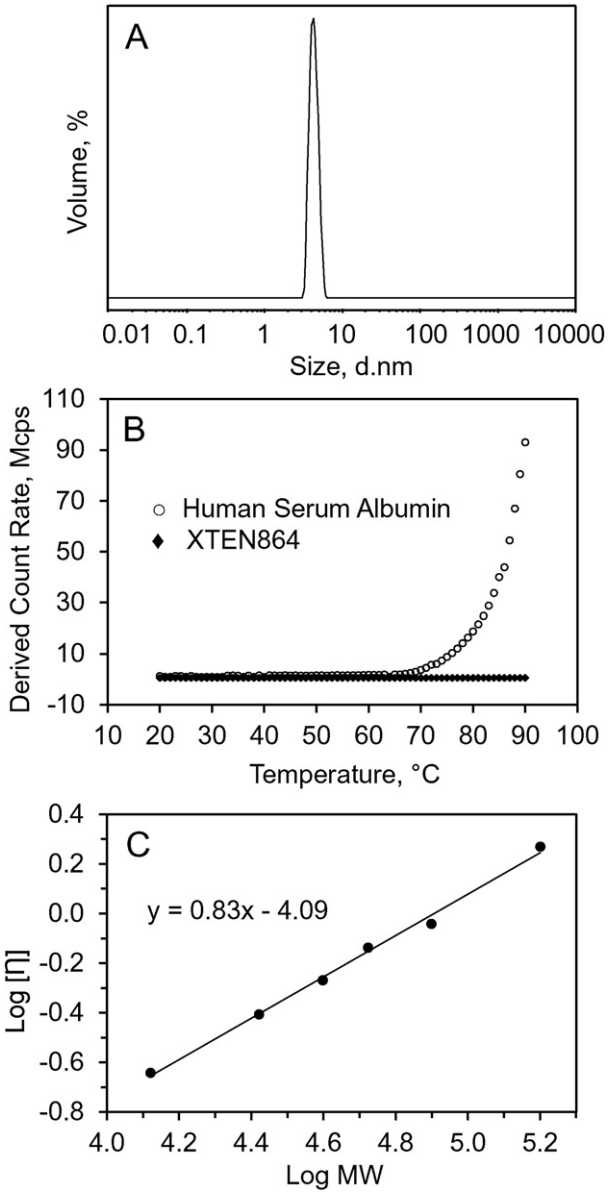

Fig. 3. DLS and viscosity characterization of XTEN polymers. Panel A: DLS volume distribution profile of XTEN864 at 100 mg/mL in phosphate buffer saline (PBS). Analysis was performed using a Zetasizer Nano S system (Malvern Instruments, Westborough MA). Panel B: DLS temperature trend graph comparing the thermal stability profiles of human serum albumin (HSA) with XTEN864, both prepared at 2 mg/mL in PBS. Panel C: intrinsic viscosity vs. molecular weight plot for XTEN144, XTEN288, XTEN432, XTEN576, XTEN864 and XTEN1728 polymers. Viscosity measurements were performed using a microVISC™ temperature-controlled viscometer (RheoSense, San Ramon CA). The slope of the line was determined by simple linear regression analysis.

2.2. Payload linking approaches

XTEN protein polymers consist of genetically encoded amino acids and thus can be recombinantly fused to therapeutic proteins or peptides. An obvious benefit of genetic fusion constructs is the convenience of expression, purification and characterization of a single molecule which includes both the therapeutic and bulking moieties (Fig. 4, A). Multiple fusion combinations are possible depending on whether the N- or C-termini of the therapeutic moieties are dispensable for biological activity. XTEN polypeptides have been fused to the C-termini of exenatide [19], glucagon (Gcg) [20], glucagon-like peptide 2 (GLP2) analogue [25], and to the N-terminus of Annexin A5 [41].

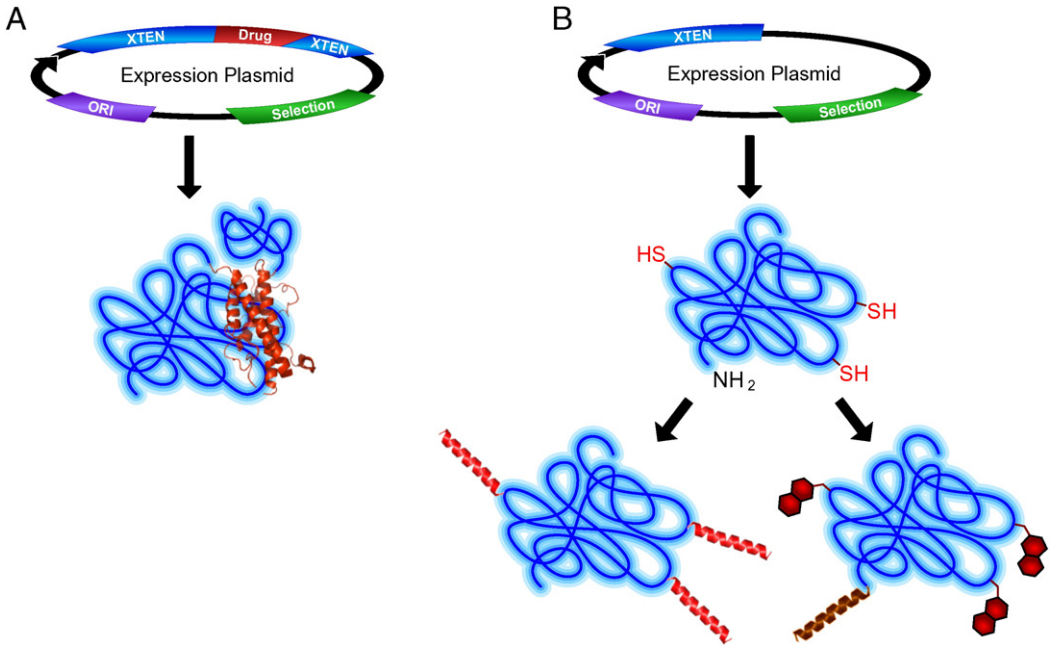

Fig. 4. XTENylation of biologically active molecules. Panel A: Genetic fusion — a single polypeptide which includes both the therapeutic and bulking moieties. Panel B: Chemical conjugation — XTEN is first expressed and purified as an intermediate and then conjugated to biologically active payloads.

A functional advantage of using genetic fusion to produce XTENylated proteins is the ability to fuse multiple XTEN fragments to a therapeutic protein of interest. This advantage has been used to enhance the therapeutic capabilities of several products we have developed. In the case of human growth hormone (hGH), rapid kidney clearance was minimized by fusion of a 912 amino acid residue sequence of XTEN to the N-terminus of the therapeutic protein, while receptor-mediated clearance was reduced by fusion of a 144 amino acid residue sequence of XTEN to the C-terminus (discussed in greater detail in Section 4.1.1). In the case of coagulation factor VIII, multiple XTEN fragments were integrated into the native sequence of the therapeutic polypeptide (discussed in greater detail in Section 4.2).

Chemical conjugation approach requires separate production of XTEN and the therapeutic payload, selective cross-linking of components, and purification of the final conjugate (Fig. 4, B). While these additional steps may seem cumbersome, chemical conjugation has distinct advantages over genetic fusion. Although XTEN fusion proteins are typically easily expressed, some scouting and optimization of the expression system and the purification steps are required. In contrast, manufactured XTEN polymers are produced in a standardized technology platform in which expression has been optimized to yield high titers, and the subsequent XTEN purification processing is efficient and readily scalable, yielding homogeneous, highly purified bulk intermediates [21,24]. To be amenable to genetic fusion, the payload molecule must be composed of proteinogenic L-amino acids, and the fusion product must be produced as a linear unidirectional polypeptide. By contrast, the chemical conjugation approach allows XTEN to be applied to a broader spectrum of payloads including peptides containing non-natural amino acids and post-translational modifications, peptoids, β-peptides, nucleic acids and other therapeutically relevant small molecules. Furthermore, conjugation allows the chemical payload to be attached in any desired orientation.

An XTEN polypeptide consisting entirely of A, G, E, P, S and T carries a single uniquely positioned reactive α-amino group. This group allows conjugation of payloads containing reactive esters such as N-hydroxysuccinimide (NHS) esters and tetra- or pentafluorophenyl (TFP, PFP) esters. Additional reactive amino groups can be introduced by incorporation of lysine(s) into selected position(s) along the XTEN polypeptide backbone by standard cloning techniques. Incorporation of cysteines into the XTEN polypeptide allows conjugation of maleimide- and haloacetyl-containing payloads. Given sufficient differences in the reactivity of amino- and thiol groups, efficient orthogonal conjugation of unrelated payloads can be accommodated. Additional chemical modifications, such as periodate oxidation of N-terminal serine [42,43] and conversion of naturally occurring reactive groups such as amines and thiols into chemically orthogonal groups such as azide, alkyne, aldehyde, alkoxyamine [44] have been demonstrated for XTEN protein polymers (Table 1).

To date, a variety of bioactive peptides have been successfully XTENylated. Despite the substantial negative charge and bulkiness of XTEN, conjugation reactions generally proceed quickly and efficiently for peptides of various sizes and charges, suggesting that the physicochemical characteristics of XTEN do not result in shielding of reactive moieties on the polymer or unproductive repulsive or attractive interactions between XTEN and the target peptide.

3. Biological properties of XTEN

3.1. Pharmacokinetics

A key attribute of the XTEN technology is the tunability of XTEN polymer length to achieve targeted half-life [19]. Longer XTEN polypeptides have larger hydrodynamic volumes (Fig. 1, A) and are expected to exhibit slower kidney clearance. Furthermore, SEC regression lines for globular proteins and XTEN series diverge as MWs increase (Fig. 1, B), indicating that larger XTEN gain more bulkiness than expected solely from MW increase. As a result, the magnitude of *in vivo* half-life gain has been observed to be more than the proportional increase in XTEN length. As shown in Table 2, XTEN864 protein polymer, which is twice the length of XTEN432, exhibits about 3-fold longer half-life than XTEN432: 25 h versus 9 h in mice; 42.9 h versus 13.5 h in rats.

While the terminal half-lives of XTEN polymers seem to be very predictable, linking a bioactive protein or peptide can substantially alter these values. To give an example, green fluorescent protein (GFP), exenatide and GLP2 analogue (GLP-2G) were each fused to XTEN864, yielding proteins with plasma half-lives in cynomolgus monkeys of 73 h, 60 h and 120 h, respectively (Tables 2, 3), likely reflecting payload-related clearance. However, the half-life of any

Table 1
Conjugation chemistries tested for XTEN protein polymers.

Payload functionality	XTEN functionality	XTEN derivatization	Conjugation product
NHS, TFP, PFP active ester	N-terminal α-amine, lysine	Not needed	Amide
Maleimide	Cysteine	Not needed	Succinimidyl thioether
Thiol	Maleimide	Maleimide-NHS ester	Succinimidyl thioether
Iodoacetyl	Cysteine	Not needed	Thioether
Thiol	Iodoacetyl	Succinimidyl iodoacetate	Thioether
Alkoxyamine	Aliphatic aldehyde	$NaIO_4$ oxidation of N-terminal serine	Oxime
Alkoxyamine	Aromatic aldehyde	4-formyl-benzamido-TFP ester	Oxime
Aldehyde	Alkoxyamine	Phthalimidooxy-NHS ester	Oxime
Azide	Alkyne	Alkyne-NHS ester	Triazole
Alkyne	Azide	Azide-NHS ester	Triazole

individual payload can be tuned by adjusting the length of the XTEN polymer, as was demonstrated by the GFP fusions to a range of XTEN lengths (e.g. XTEN288 to XTEN864). The variation in half-life was XTEN length-dependent, ranging from 8 h to 29 h in rats and 22 h to 72 h in cynomolgus monkeys (Table 2). Half-life extension has been observed in all XTEN-modified peptides and proteins analyzed to date, including exenatide, GLP2-2G, Gcg, hGH, and coagulant factors (Table 3, Section 4.2).

Half-life extension of therapeutic moieties leads to increased *in vivo* exposure to the pharmaceuticals and maintenance of plasma concentrations within the therapeutic window. Although extended half-lives may be gained at the expense of decreased *in vitro* activity, the reduction in many cases is compensated for by prolonged *in vivo* exposure, resulting in improved efficacy comparable to the unmodified payload at equimolar doses. Another important feature of XTEN-modified therapeutics is their relatively flat peak-to-trough ratio following subcutaneous administration, a characteristic particularly helpful in preventing spikes in drug plasma concentration that can lead to toxicity and other unfavorable side effects [19].

3.2. Biodegradation

To gauge the biodegradability of XTEN, purified XTEN864 was incubated in rat plasma, rat kidney homogenate or PBS (Fig. 5). XTEN polymer was found to be stable in plasma, with a degree of proteolysis comparable to random hydrolysis in buffer. In contrast, XTEN was rapidly and completely degraded in cell homogenate to low molecular weight species with no stable intermediates. Similar observations were made using GFP-XTEN fusion detected by Western blot using anti-GFP antibody [19]. Rapid degradation of XTEN by intracellular proteases implies that the protein polymer will not accumulate in tissues following prolonged medication.

3.3. Biodistribution

The biodistribution of the 79 kDa XTEN864 protein polymer has been investigated in Sprague Dawley rats. Animals were intravenously injected with XTEN864 labeled with a tag consisting of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) coupled to a lanthanide ion. At predetermined time points, biological samples were collected and analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) [45,46]. By day 14 (~8 half-lives) XTEN864 was cleared from circulation and the combined amount of XTEN detected in all organs totaled less than 2% of the total initial injected dose (ID). The residual distribution of XTEN remaining in individual organs is shown in Fig. 6, A. Accumulation in brain tissue was particularly low, being below the limit of detection at <0.002% ID per g tissue. XTEN864 was eliminated primarily via the kidney. Quantitation of metal in urine accounted for 7.67% ± 1.2% ID within the 48 to 72 h collection time frame. Feces accounted for only 1.35% ± 0.12% ID in the same time frame. The sum of XTEN amounts excreted in urine and feces correlated with the amount of XTEN removed from the plasma (Δm_{plasma}), 11.4 ± 1.67% ID, during the same time (Fig. 6, B).

Biodistribution profiles of the 79 kDa XTEN864 and 26 kDa XTEN288 polymers were also investigated in Swiss Webster mice. Both XTEN864 and XTEN288 were labeled, each with a unique lanthanide metal via the DOTA chelator. Labeled XTEN was injected intravenously into mice as a mixture. Plasma samples were collected at predetermined time points from pre-dose to 36 h; organs were harvested at 8 h and 36 h. All samples were analyzed by ICP-MS. XTEN288, with a short half-life of less than 2.5 h (Table 2), was rapidly cleared from both plasma and the vital organs that were examined. XTEN864 was detected in the plasma at high concentrations at both time points due to its much longer half-life of 25 h (Table 2). While XTEN864 was able to penetrate all organs except brain via the vasculature, accumulation in organ tissue was not observed. Organ concentrations were consistent with blood concentration, likely due to plasma perfusion to the organs. These data demonstrate that XTEN polymers accessed most tissues via the bloodstream, but did not accumulate and were cleared from tissues when plasma levels of XTEN decreased (Fig. 7).

This non-accumulating biodistribution profile of XTEN in healthy tissue together with its rapid biodegradability by intracellular proteases suggests that XTEN may be a safer alternative to PEG for half-life extension of pharmaceuticals.

Table 2
Terminal plasma half-lives of XTEN protein polymers.

XTEN	Species	Half-life, h	Bioanalytical method
XTEN864	Rat	42.9	ICP-MS [45,46]
XTEN576	Rat	22.6	ICP-MS
XTEN432	Rat	13.5	ICP-MS
XTEN864	Mouse	25.0	ICP-MS
XTEN432	Mouse	9.0	ICP-MS
XTEN288	Mouse	<2.5	ICP-MS
GFP-XTEN864	Rat	29	Anti-GFP ELISA [19]
GFP-XTEN576	Rat	15	Anti-GFP ELISA
GFP-XTEN288	Rat	8	Anti-GFP ELISA
GFP-XTEN864	Monkey	73	Anti-GFP ELISA
GFP-XTEN576	Monkey	22	Anti-GFP ELISA

3.4. Immunogenicity

BLAST analysis of established XTEN sequences shows that XTEN864 does not possess significant homology to naturally occurring human proteins. A scan for T cell epitopes using the ProPred MHC class II binding peptide predictor [59] against the most prevalent HLA DR alleles revealed a complete lack of T cell epitopes (P1 anchors) within XTEN (data not shown). Attempts to generate an anti-XTEN antibody for bioanalytical purposes have proved to be extremely challenging. After much effort, only a single stable low affinity anti-XTEN antibody was obtained. This was achieved by hyper-immunizing mice with exenatide-XTEN protein in the presence of complete Freund's adjuvant. The resultant anti-XTEN antibody was characterized to be specific for XTEN and not for exenatide.

Table 3
Fold improvement in half-lives of XTEN-modified therapeutics.

Payload	Species	$t_{1/2}$ of payload, h	XTEN length	$t_{1/2}$ of XTENylated payload, h	$t_{1/2}$ of XTENylated payload/$t_{1/2}$ of payload
Exenatide	Human	2.4 [47]	864	124 [48]	52
Exenatide	Mouse	0.17 [49]	864	12 [19]	71
Exenatide	Rat	0.49 [49]	864	32 [19]	65
Exenatide	Monkey	0.48 [50]	864	60 [19]	125
Exenatide	Dog	ND	864	72.8[a]	ND
GLP2-2G	Human	1.6–2.2 [51]	864	Predicted 240 [25]	109 to 150
GLP2-2G	Mouse	0.37–0.58 [51]	864	34 [25]	59 to 92
GLP2-2G	Rat	0.41–0.67 [51]	864	38 [25]	57 to 93
GLP2-2G	Monkey	1.4 to 1.9 [51]	864	120 [25]	63 to 86
hGH	Human	2–3 [52,53]	912 + 144	131 [54]	44 to 66
hGH	Rat	0.53 [55]	912 + 144	15 6 [56]	28
hGH	Rat	0.53 [55]	912	6.8 6 [56]	13
hGH	Monkey	2 to 3 [57]	912 + 144	110 6 [56]	37 to 55
hGH	Monkey	2 to 3 [57]	912	48.6 6 [56]	16 to 24
Glucagon	Monkey	ND	288	9 [20]	ND
Glucagon	Monkey	ND	144	2.7[a]	ND
Fuzeon	Rat	2.8 [58]	432	55.7 [21]	20

ND, not determined.
[a] Unpublished data.

Preclinical and clinical immunogenicity profiles of XTEN were evaluated through two XTEN-modified pharmaceuticals, VRS-859 (XTENylated exenatide) and VRS-317 (XTENylated hGH). Preclinical pharmacokinetics and toxicity of VRS-859 (discussed in greater detail in 4.1.2) were evaluated in mice and cynomolgus monkeys. Mice were dosed up to 50 mg/kg every other day for 29 days followed by a 2-week recovery period; monkeys were dosed up to 35 mg/kg weekly for 4 weeks followed by a 2-week recovery period. In both animal species, VRS-859 was found to be well-tolerated and generally non-immunogenic with no adverse effect observed at highest doses tested [60]. Results from a Phase I, single-ascending dose study designed to evaluate the safety and tolerability of VRS-859 demonstrated that VRS-859 in up to 200 mg dose was well tolerated with no unexpected adverse events [48].

The most advanced immunogenicity evaluation of XTEN has been performed with VRS-317, an XTENylated hGH currently in Phase III clinical testing in children with GHD (discussed in greater detail in Section 4.1.1). In a preclinical repeat dosing study involving juvenile cynomolgus monkeys, VRS-317 was dosed at 0.4 and 1.4 mg/kg every 28 days for 84 days with 4 animals per dose group. Clinical pathology performed on pre-dose and various post-dose time points revealed no significant changes in clinical chemistry and hematology. An immunogenicity evaluation revealed that two monkeys in the 1.4 mg/kg dose group developed low titer antibody to VRS-317 at day 85. The antibody was found to be specific to hGH but not the XTEN domain; and was attributed to the lack of homology between cynomolgus GH and rhGH. Importantly, the pharmacokinetics and pharmacodynamics profiles were not affected, suggesting that the low titer antibody response is unlikely to have a significant clinical effect [56]. Phase Ia immunogenicity evaluation of VRS-317 demonstrated that 4 out of 40

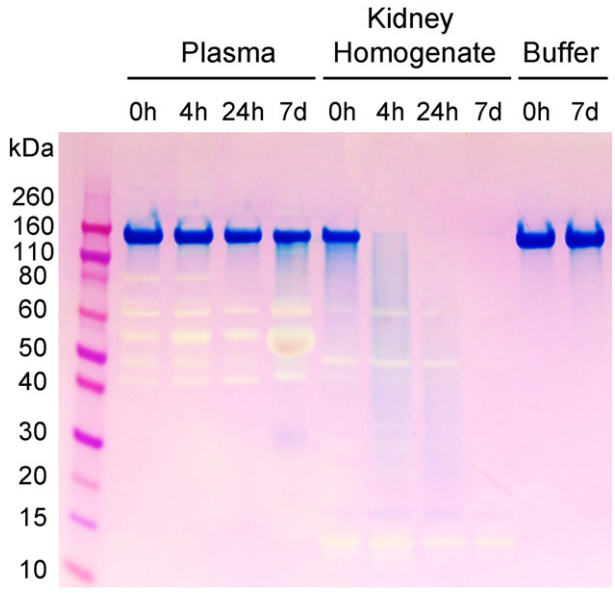

Fig. 5. Biodegradation of XTEN864. XTEN was added to rat plasma, rat kidney homogenate, or PBS to a final concentration 0.3 mg/mL and incubated at 37 °C, then mixed with two volumes of cold methanol. Precipitated media proteins were removed by centrifugation. Supernatants were concentrated by evaporation, redissolved in loading buffer and analyzed by SDS-PAGE. Proteins were visualized by Stains-All dye. The full-length XTEN864 migrates on the gels at the same rate as the 160 kDa calibrant.

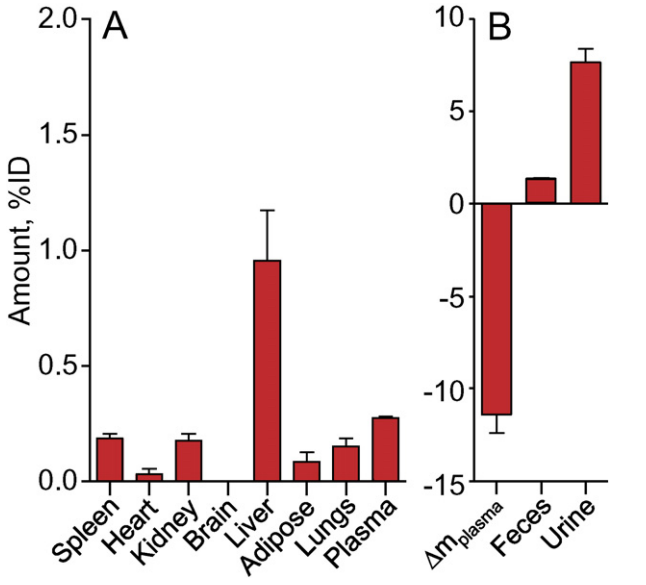

Fig. 6. Lack of accumulation of XTEN864 in body organs. Panel A: XTEN864 polymer was labeled at the N-terminal α-amino group with DOTA-NHS ester (Macrocyclics, Dallas TX), then labeled with lanthanide metal and injected intravenously into rats. At predetermined time points, plasma, feces and urine were collected. Additionally, various organs such as brain, spleen, heart, kidney, liver, mesenteric adipose tissue and lungs were harvested. All samples were analyzed by ICP-MS to determine the concentration of the lanthanide metal label [45,46]. Panel B: plasma samples were taken at 48 and 72 h post injection and analyzed for metal content to determine Δm_{plasma} value. Excrement samples were collected between 48 and 72 h post injection and analyzed for metal content to evaluate the XTEN elimination route.

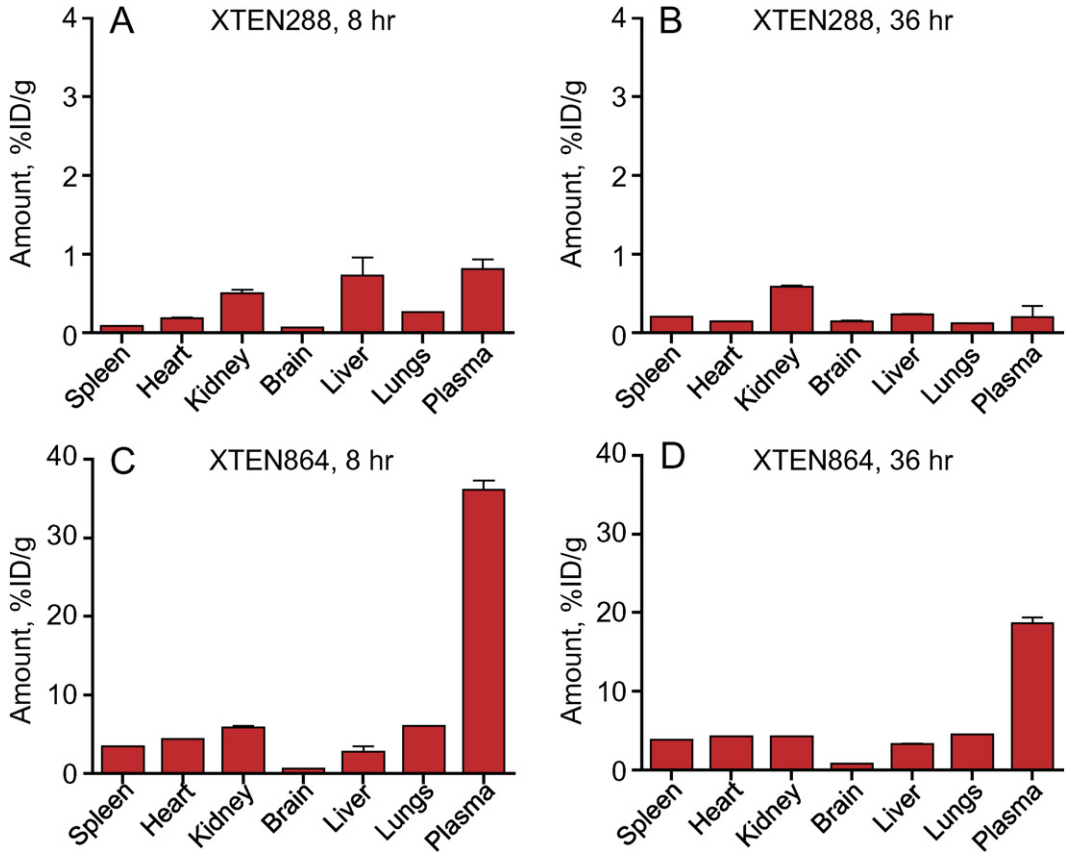

Fig. 7. Non-accumulation of XTEN288 and XTEN864 in normal mouse tissues. XTEN864 and XTEN288 were labeled with lanthanide metal DOTA tags and injected into mice. At 8 h and 36 h time points, the animals were sacrificed and the organs were analyzed for levels of the lanthanide metal labels.

GHD adult patients generated a low titer anti-VRS-317 antibody response at 30 days and/or 60 days after VRS-317 dosing. Three of the 4 adult GHD patients had an antibody titer of 1:5 and one patient a titer of 1:25. However, upon further analysis in an anti-hGH antibody assay, antibody detected in 3 of the 4 patients was found to be nonspecific [54]. Subsequent Phase Ib/IIa and Phase IIb extension studies in pre-pubescent children with GHD all demonstrated that VRS-317 is well-tolerated with no unexpected adverse events [61–63].Taken together, existing clinical data suggest that XTEN has low or no immunogenicity in humans.

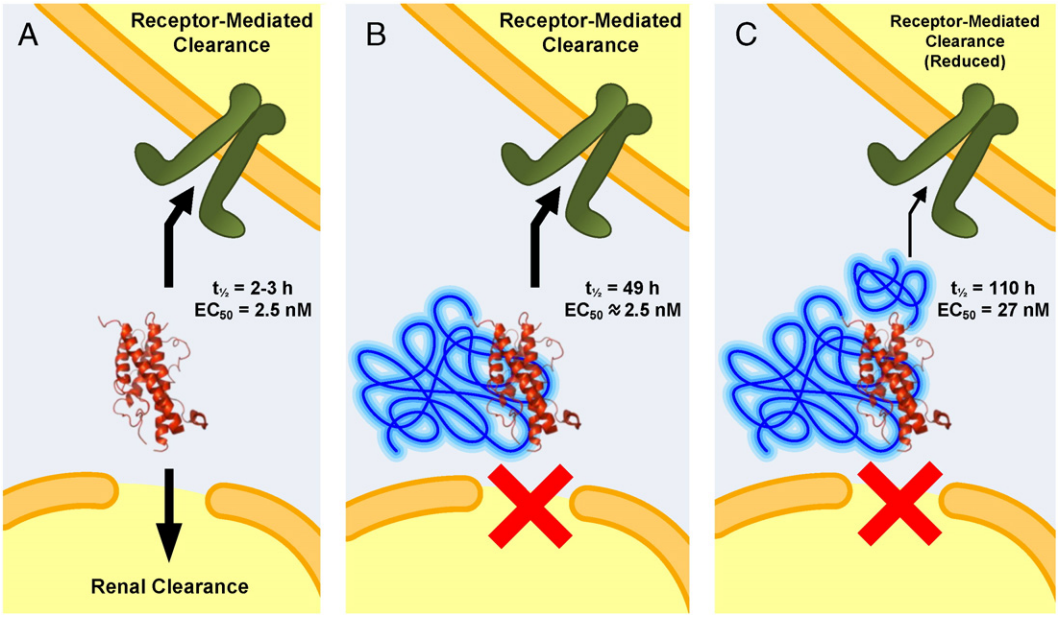

Fig. 8. Dual inhibition of hGH clearance by XTEN. Panel A: native hGH, cleared by 2 routes with terminal half-life 2–3 h in monkeys [57]. Panel B: N-terminal XTEN912 fusion to hGH reduces renal clearance (half-life in monkeys 49 h [56]). Panel C: VRS-317 (hGH with XTEN at C- and N-termini) reduces both renal and receptor-mediated clearance (half-life in monkeys 110 h [56]). Binding affinity was measured using an ELISA-based receptor binding assay [56].

4. XTEN applications

4.1. Half-life extension by genetic fusion to peptides and proteins

4.1.1. Human growth hormone

Current hGH replacement therapy for children with GHD requires daily injections of recombinant hGH over a number of years. Maintaining full treatment compliance is challenging for many young patients, and dose omissions are found to occur frequently [64–67]. Lack of compliance with daily hGH therapy has been associated with statistically significant diminished growth velocities [68]. Thus, a long acting hGH product to reduce dosing frequency and improve compliance and overall treatment outcomes would be highly desirable.

Human growth hormone is eliminated from the body by both kidney filtration and receptor-mediated clearance (Fig. 8), resulting in a short half-life of the drug of 2–3 h in monkeys. VRS-317, a novel, long-acting hGH-XTEN fusion protein under clinical development for the treatment of GHD, was specifically designed to encompass such dual clearance inhibition mechanism, and thereby achieving an extended $t_{1/2}$ of 15 h in rats and 110 h in monkeys [56]. Despite a measured 12-fold reduction of *in vitro* activity compared to native hGH, the enhanced *in vivo* exposure to target tissues and organs compensates for the loss with VRS-317 attaining a more than a 3- and 5-fold gain in efficacy compared to daily hGH in hypophysectomized rats and juvenile monkeys respectively [56].

Clinical evaluation of VRS-317 was initiated in 2012 in 50 GHD adults. In this single ascending dose study, VRS-317 was found to be efficacious, well-tolerated with no unexpected or serious adverse events. A half-life was determined to be 131 h, highlighting VRS-317 as a potential monthly dose therapy [54]. This study was followed by Phase Ib/IIa trials in treatment naïve pre-pubescent GHD children with a subcutaneous dosing regimen of 5 mg/kg monthly, 2.5 mg/kg semi-monthly and 1.25 mg/kg weekly. Upon achieving positive efficacy as determined by annualized height velocity and safety endpoints, the Phase IIa study was extended for an additional 6 months to a Phase IIb with 5 mg/kg monthly, 2.5 mg/kg semi-monthly and the 1.25 mg/kg weekly arm transitioned to a 3.5 mg/kg semi-monthly arm. The rate of adverse events was lower in the second 6 months of the trial and safety was comparable to daily GH treatment. Insulin-like growth factor 1 (IGF-1) levels in the 3.5 mg/kg semi-monthly dose cohort were almost a full standard deviation (SD) higher than the mean IGF-1 SD experienced by the 2.5 mg/kg semi-monthly cohort. In addition, a subset of the 3.5 mg/kg semi-monthly cohort recorded an increased mean annualized height gain from 7.5 cm/year in the first 6 months to 9.3 cm/year during the second 6 months of the study. The mean height velocity measured after 12 months of continuous VRS-317 therapy for patients who remained on the 5 mg/kg monthly and 2.5 mg/kg semi-monthly doses in the extension study was determined to be not significantly different from the mean height velocity observed in the same patients at 3, 6 and 9 months. In addition, the mean height velocity for patients completing 12 months of treatment at 2.5 mg/kg semi-monthly was 8.5 cm/year, consistent with the first year growth rate for moderate GHD patients receiving the highest approved dose of hGH [63]. Following positive outcomes from the Phase I and II studies, a Phase III study has been initiated in US, Europe and Canada, with a primary endpoint of non-inferiority for 12 month height velocity between 3.5 mg/kg VRS-317 dosed twice-monthly and control group receiving daily hGH dosing [69]. A Phase 2/3 study has also commenced in Japan [70].

4.1.2. Exenatide

Type 2 diabetes (T2D) constitutes approximately 90–95% of all diabetes cases in US, with aging, family history, obesity and physical inactivity being the major causes [71,72]. Exenatide is a 39 amino-acid peptide with effects similar to glucagon-like peptide 1 (GLP-1), currently marketed as Byetta for the treatment of T2D. Owing to its small size and rapid renal clearance, Byetta has a plasma half-life of 2.4 h and is dosed twice daily. The most common reported side effects associated with Byetta therapy are nausea, vomiting and diarrhea [47]. In 2012, Bydureon, a slow release version of Byetta, was approved by the FDA as the first once-weekly GLP-1 medicine for T2D. However, Bydureon suffers from a high incidence of injection-site nodules [73].

To extend the circulating half-life of exenatide, XTEN864 was fused to the C-terminus of the peptide [19]. The fusion molecule is known as exenatide-rPEG or VRS-859 [74]. VRS-859 was extensively tested in animal models, and based on allometric scaling from pharmacokinetic parameters determined in mice, rats, monkeys, and dogs, a half-life of 139 h was predicted in human. As is characteristic of XTEN fusion proteins, VRS-859 exhibits a slow absorption phase and a relatively flat peak before reaching a linear elimination phase following subcutaneous injection into monkeys [19]). This pharmacokinetic profile has been postulated to decrease the severity of adverse effects caused by the sharp peak-to-trough toxicity observed for native exenatide. In a series of pharmacodynamics studies in animal models, VRS-859 was found to be efficacious in sustaining glycemic control and imparting weight loss [60].

A Phase I multi-center, blinded, placebo-controlled, single-ascending dose study to evaluate the safety and tolerability of VRS-859 in 70 T2D patients was initiated in 2010. Positive Phase I results demonstrated that all doses administered (up to 200 mg) were well tolerated and able to provide glycemic control in T2D patients. Gastrointestinal adverse events were generally mild and transient and resolved in less than 24 h. Pharmacokinetics of VRS-859 was dose-linear and the half-life was determined to be 128 h, comparable to preclinical PK prediction by allometric scaling. The data suggest that VRS-859 has the potential ability to maintain glycemic control for one month in T2D patients after a single dose [48].

4.1.3. GLP2

Patients suffering from short bowel syndrome, a malabsorption disorder caused by the surgical removal of some portion of the small intestine due to primary causes such as cancer or Crohn's disease, can result in the life-long dependence on parenteral nutrition, which greatly reduces quality of life. Teduglutide, marketed as Gattex in the United States and Revestive in Europe, is a glucagon-like peptide 2 (GLP2) analogue indicated for the treatment of adult patients with short bowel syndrome. Bearing a glycine substitution at position 2, teduglutide (GLP2-2G) has increased half-life and stability as compared to the native GLP2, prolonging half-life from 7 min to 3 to 5 h in human [75–77]. However, even with this gain in circulating half-life, teduglutide needs to be dosed daily. A sustained exposure with a long-acting GLP2-2G-XTEN should lead to superior efficacy, less frequent dosing and enhanced dosing adherence.

GLP2-2G-XTEN was produced by the genetic fusion of the GLP2-2G peptide to an 864 amino acid-long XTEN at its C-terminus [25]. The plasma half-life of GLP2-2G-XTEN was significantly extended to 34 h, 38 h and 120 h in mice, rats and monkeys respectively. Based on allometric scaling, a half-life of 240 h in human was estimated, suggesting the feasibility of a monthly dosing regimen in humans. When compared to GLP2-2G peptide in a rat Crohn's disease model, GLP2-2G-XTEN was found to require a lower molar dose and less frequent dosing to achieve equivalent intestinotropic effects as GLP2-2G. Prophylactic administration of GLP2-2G-XTEN led to significant increase in length, reduction in number of trans-ulcerations and adhesions and TNF-α content of the small intestine. These improvements in preclinical pharmacokinetics, pharmacodynamics and dosing indicate that GLP2-2G-XTEN may offer superior therapeutic benefit for the treatment of short bowel syndrome and other gastrointestinal diseases such as Crohn's disease [25].

4.1.4. Glucagon

Hypoglycemia is the most common complication of diabetes therapy, affecting 90% of the diabetic population [78–80], with patients who experience severe hypoglycemia having an increased mortality risk in

comparison to those not experiencing severe hypoglycemia [81–84]. Gcg, a peptide hormone capable of converting hepatic glycogen stores into glucose for release into the bloodstream, is often administered in such cases of acute hypoglycemic crisis. Due to its poor solubility and stability in liquid formulation, currently marketed Gcg is supplied as lyophilized powder requiring reconstitution [85].

While hypoglycemic events during the day can be recognized and treated promptly, nocturnal hypoglycemia poses a more significant health risk, especially in juvenile type 1 diabetic patients where the incidence of nocturnal hypoglycemic episodes is particularly high [86]. Native Gcg has an extremely short half-life of 8 to 18 min [85] and is thus unsuitable for prophylactic use in nocturnal hypoglycemia. As the XTEN polypeptide has the propensity to improve solubility, stability and half-life of the attached peptide, an XTEN-modified Gcg may prove useful for the potential prophylactic treatment of nocturnal hypoglycemia.

An XTENylated Gcg was generated by the recombinant fusion of 144 amino acid-long XTEN to the C-terminus of Gcg [20]. Gcg-XTEN144 was observed to have a 60-fold better solubility profile than native glucagon, allowing for liquid formulation. In a fasted beagle dog model, both Gcg-XTEN144 and native Gcg were found to induce a rapid increase in blood glucose. However, only the effect of Gcg-XTEN144 was sustained for 10–12 h whereas the effect of Gcg returned to baseline by 2 h. Importantly, prophylactic administration of 0.6 nmol/kg Gcg-XTEN144 to fasted dogs conferred strong resistance to insulin-induced hypoglycemic challenge at 6 h post dose (mimicking a nocturnal hypoglycemic episode) but not at 12 h post-dose (mimicking waking cycle) without affecting baseline blood glucose levels [20]. These data suggest that Gcg-XTEN144 has the potential to be an effective therapeutic in the prevention of nocturnal hypoglycemia.

4.2. XTEN fusions to clotting factors

Hemophilia A and B are X-linked bleeding disorders caused by a deficiency in either clotting factor VIII (FVIII) or factor IX (FIX) activity, respectively. The standard treatment for hemophilia has evolved over the years to the current preferred prophylaxis regimens with regular infusions of either plasma-derived or recombinant FVIII/FIX to replace the missing factor, and maintain a certain amount of clotting activity in the blood to prevent bleeds before they occur [87]. These prophylaxis regimens have resulted in significant improvements in clinical outcomes, but have a significant treatment burden due to the short half-lives of the factors, such that hemophilia A patients on a prophylaxis regimen infuse from three times per week to every other day with FVIII, and hemophilia B patients infuse two to three times per week with FIX [88]. In cases where hemophilia patients develop neutralizing antibodies to FVIII (approximately 30%) or FIX (approximately 3%), a recombinant, activated form of factor VII (FVII) is often used (rFVIIa) to bypass the missing factor to treat bleeds, however rFVIIa has an even shorter half-life such that it must be infused every 2 h to control a bleed, and effective prophylaxis cannot be generally achieved [89]. While there have been advances in the generation of longer acting FVIII and FIX molecules that reduce the frequency of intravenous infusions [90], there remains a significant unmet medical need for either further half-life extension, and/or to enable alternate, less invasive routes of administration, such as by subcutaneous injection. With these two goals in mind, XTEN fusion technology was applied to all three clotting factors, initially through C-terminal fusions.

Clotting factor IX is an approximately 55 kDa vitamin K-dependent acid serine protease with a number of key post-translational modifications such as gamma-carboxylation that require it to be generated in mammalian cells [91]. FIX was first generated as a C-terminal XTEN864 fusion protein, expressed in a HEK cell line, purified by conventional chromatography methods, and pharmacokinetics determined by assessing the FIX activity at various time points after single intravenous infusion in hemophilia B mice (Fig. 9, A) [92]. The simple XTEN C-terminal fusion resulted in significant extension of half-life in comparison to rFIX, with a terminal half-life of 40 h, compared to an approximately 15 hour terminal half-life for rFIX. Interestingly, the XTEN fusion also increased the plasma recovery significantly compared to rFIX (82% v. 20%), similar to the results observed with FIX that was PEGylated on the N-glycans of the FIX activation peptide [93]. This is consistent with the similar mechanism of action as PEGylation, related to increased hydrodynamic radius and possible inhibition of clearance receptor binding or protease cleavage, but in the case of XTEN fusion, with the added advantages of being fully recombinant without requiring any additional chemical modification, and ultimately being metabolized *in vivo* due to a composition of solely native amino acids. These results demonstrated that XTEN fusions could be efficiently produced in mammalian cell culture and result in significant half-life extension of a complex biologic compared to the unmodified protein.

Clotting factor VII is an approximately 45 kDa serine protease that also requires posttranslational modifications such as gamma carboxylation, and therefore was generated in mammalian cells in a similar manner to FIX. In contrast to therapeutic FIX, the therapeutic FVII is administered as an active protease, and therefore rFVIIa variants require an additional activation step during production. FVII was first generated as a C-terminal XTEN288 fusion protein, purified by conventional chromatography methods, activated by incubation overnight at high protein concentration in the presence of calcium, and the pharmacokinetics determined by assessing the soluble tissue factor prothrombin-time (sTF PT) activity at various time points after single intravenous infusion in hemophilia A mice (Fig. 9, B) [94]. Here also a significant terminal half-life extension was observed compared to rFVIIa (8.9 h vs 1.2 h), as well as significant increases in recovery and area under the pharmacokinetic curve (46 vs 9.2 h*kg/L). As with FIX, these results demonstrate that XTEN fusion can be successfully applied to a complex biologic and result in significant improvements in pharmacokinetic parameters.

Clotting factor VIII is an approximately 280 kDa protein that mediates multiple protein:protein and protein:phospholipid interactions in its function as a cofactor [95]. Deletion of a large portion of the central B-domain (BDD, B domain deleted) reduces the size of FVIII to 170 kDa and has no effect on the functional activity of FVIII, but increases expression levels in eukaryotic cells [96]. The majority of FVIII in circulation (95–98%) is normally complexed with von Willebrand Factor (VWF), a large glycoprotein approximately 250 kDa in size that forms multimers up to 20 MDa, which stabilizes FVIII and protects it from premature cleavage or clearance [95]. BDD FVIII was initially expressed as a C-terminal XTEN864 fusion protein, purified by affinity and ion exchange chromatography, and the pharmacokinetics determined by assessing the FVIII activity at various time points after a single intravenous infusion in hemophilia A mice (Fig. 9, C). FVIII-XTEN exhibited an approximately 2 fold extension of half-life in hemophilia A mice compared to B domain-deleted FVIII alone (Tongyao Liu, unpublished data). While this degree of half-life extension was lower than seen with clotting factors IX and VIIa, it is similar to that observed with a number of other technologies applied to FVIII in preclinical studies [90]. This is consistent with the postulate that the VWF association, while providing stability to FVIII, also provides a half-life limitation on FVIII, due to coupling FVIII to the VWF clearance pathways that result in VWF having a half-life on the order of 16 h. In order to get a better assessment of the half-life extension of FVIII as an XTEN fusion, these pharmacokinetic studies were repeated in double knockout mice (DKO) in which both the FVIII and VWF genes were deleted (Fig. 9, D) (Tongyao Liu, unpublished data). As expected, the rFVIII control had a much lower half-life in the absence of VWF (0.23 h in DKO vs 7.6 h in HemA), however the FVIII-XTEN demonstrated a much longer half-life extension in these mice compared to FVIII alone ($t_{1/2} = 7.8$ h, a 34-fold increase) with the VWF limitation removed.

In all of these studies, the initial work focused on a simple C-terminal XTEN fusion, however one significant advantage of XTEN over other

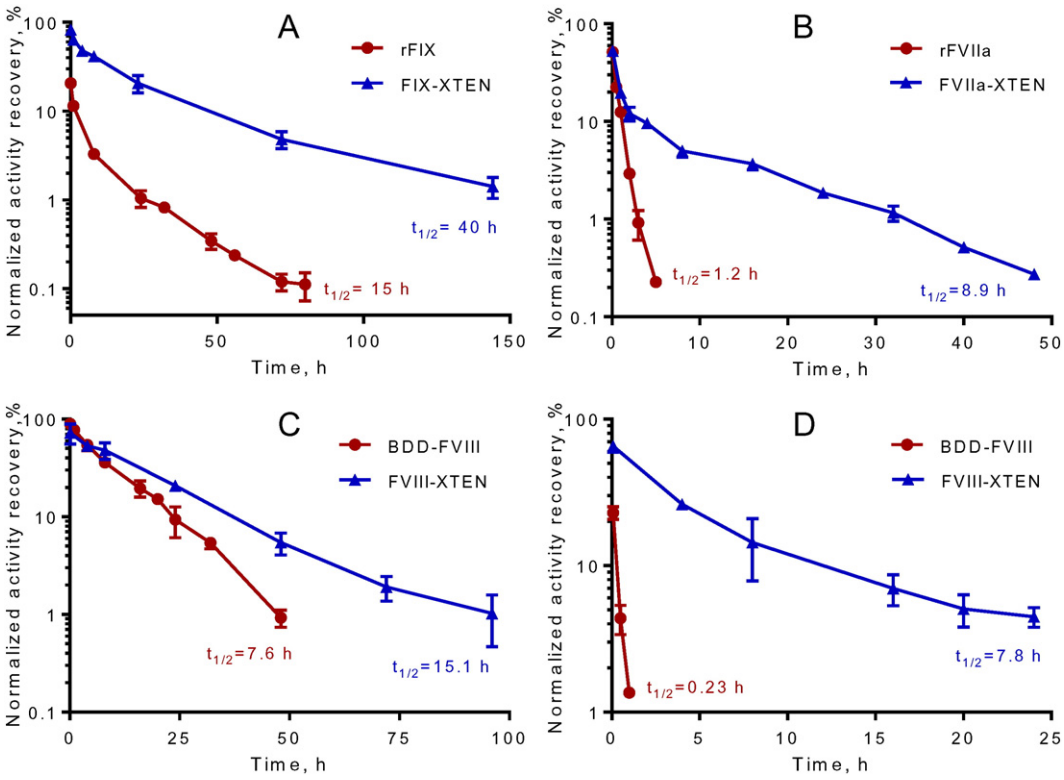

Fig. 9. Pharmacokinetics of clotting factor-XTEN C-terminal fusion proteins. In order to incorporate correct posttranslational modifications, all fusion proteins were expressed in HEK cells and purified. Activity was measured after intravenous dosing. Panel A: FIX-XTEN864 compared to rFIX in FIX-deficient (HemB) mice. Panel B: FVIIa-XTEN288 compared to rFVIIa in FVIII-deficient (Hem A) mice. Panels C and D: FVIII-XTEN864 compared to FVIII in HemA and FVIII/VWF double knockout (DKO) mice, respectively.

half-life technologies is the recombinant nature of XTEN, which allows for precise control of the placement, composition, and length of the XTEN insertions, including multiple insertions, without chemical modification. Guided by this capability, multiple sites for XTEN insertion into FVIII were chosen based on analyses of vertebrate sequences and multiple structures, and avoiding sites proximal to missense mutations associated with hemophilia A [97]. A large number of BDD FVIII variants with single 42 amino acid-long XTEN insertions were generated in mammalian cells and the FVIII activity of the conditioned media assessed as a combined measure of protein production and activity (Fig. 10, A). Based on the analysis of these results, additional sites were selected, and a number of BDD FVIII variants with single XTEN144 insertions were generated and activity assessed (Fig. 10, B). Interestingly, while the cofactor activity of FVIII requires extensive protein–protein interactions, a large number of XTEN insertion sites were identified that retained significant activity (Fig. 10).

The pharmacokinetics of a subset of single XTEN144 insertions was then assessed by activity in DKO mice following intravenous infusion [98] (Fig. 11, A). All single XTEN144 insertions demonstrated significant half-life extension of the FVIII control (0.24 h), with half-lives ranging from 2.4 to 4.5 h. Subsequent studies were then performed combining multiple intra-domain XTEN144 insertions with B-domain and C-terminal XTEN fusions to determine half-life in DKO mice. In general, increased numbers of XTEN insertions resulted in progressively greater half-life extension, well beyond those achieved with single C-terminal or intra-domain insertions (Fig. 11, B). Interestingly, assessment of single, double, or triple XTEN insertions in HemA mice all demonstrated similar half-life extension, consistent with the theory that VWF provides an upper limit on the half-life extension achievable in HemA mice. However, these results demonstrate the versatility of the XTEN technology in enabling multiple insertions into a complex biologic such as FVIII without compromising activity, and the findings have set the basis for further work in combination with other technologies to extend the half-life of FVIII beyond the VWF limit [99].

4.3. Mono- and bi-specific conjugation

GLP2-2G peptide was the first payload chemically conjugated to an XTEN protein polymer [24]. Two conjugation methods were tested. First, the α-amino group of XTEN was modified to maleimide using sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC). The activated XTEN was then conjugated to the peptide containing the C-terminal cysteine. In a second method, the peptide carrying a 3-maleimidopropionamide group at the C-terminus was conjugated to an XTEN polymer containing a single cysteine. In comparison to recombinantly fused GLP2-2G-XTEN [25], chemically conjugated GLP2-2G-XTEN exhibited similar activity and plasma stability *in vitro*. The *in vivo* half-life of the GLP2-2G-XTEN conjugate dosed subcutaneously in rats was 38.5 h, essentially the same as the 38 h half-life reported for the recombinant GLP2-2G-XTEN [25]; both values are more than 60-fold higher than the half-life in rats for the GLP2-2G peptide itself [51].

The feasibility and benefits of multivalent payload-XTEN conjugation were investigated using antiretroviral peptide T-20 (alias Fuzeon, enfuvirtide) [21]. The 3-maleimidopropionamide-T-20 peptide was conjugated to a series of XTEN polymers containing one or three cysteines with different spacing between reactive amino acids. While the T-20 peptide itself is known to have extremely poor solubility in aqueous media, the purified 1x- and 3xT-20-XTEN conjugates were soluble with no sign of aggregation. In a cell-based antiviral MAGI assay, T-20-XTEN conjugates retained antiviral activity, which could be tuned by varying the payload number as well as the positions of the peptides along the XTEN polymer. The pharmacokinetics of the most active 3xT-20-XTEN-conjugate was assayed in rats. The calculated elimination half-life of subcutaneously injected conjugate was 55.7 ± 17.7 h, about 20 times longer than the half-life reported for T-20 peptide [21].

Bi-specific conjugation of biologically active peptides to XTEN protein polymer was demonstrated for Gcg and glucagon-like peptide-1 (GLP1) analogue [100]. N-terminal α-amines of 1x- and 3x-Thiol-

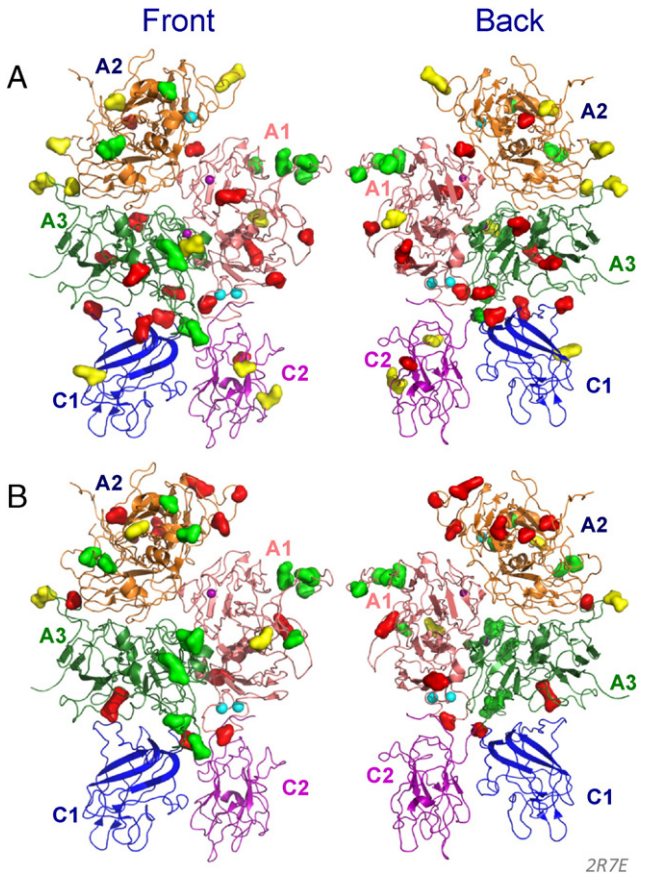

Fig. 10. Activities of FVIII-XTEN constructs. XTEN42 (Panel A) and XTEN144 (Panel B) were inserted at positions indicated. Surface representations in the 2R7E FVIII structure indicate residues immediately preceding sites of XTEN insertions, with green indicating approximately normal activity (≥0.1 IU/mL), yellow indicating reduced activity (<0.1 IU/mL), and red indicating no detectable activity.

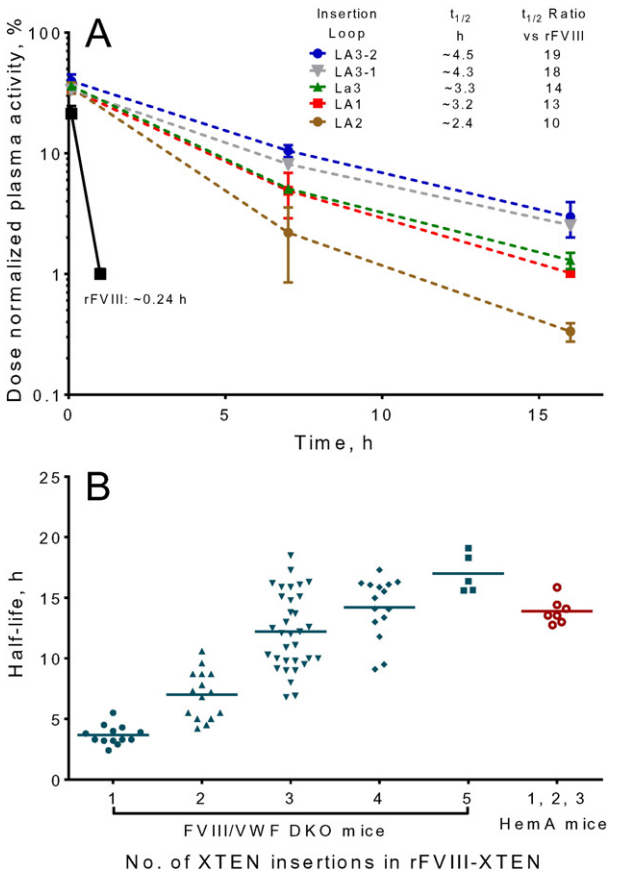

Fig. 11. Pharmacokinetics of FVIII with single or multiple intradomain XTEN insertions in FVIII-deficient (HemA) or FVIII/VWF double knockout (DKO) mice. Panel A: FVIII variants with a single XTEN 144 insertion in the indicated domain were assessed compared to FVIII alone in DKO mice. Panel B: FVIII variants with the indicated total number of different intra-domain insertions, combined with B-domain and C-terminal insertions, were assessed in DKO and HemA mice.

XTEN were modified with a dibenzocyclooctyne (DBCO) group. Gcg peptide was prepared with a C-terminal azido group, while the GLP1 analogue was modified with iodoacetyl group at the C-terminus. Using copper-free click chemistry in combination with thiol/haloacetyl chemistry, dual agonist molecules were successfully synthesized. The activities of the conjugates were tested both in cell-based assays and in diet-induced obese mice. Treatment of animals with the Gcg-XTEN-GLP1 XTEN conjugates demonstrated a synergistic effect on weight loss without compromising glycemic control [100].

5. XTEN manufacturability

The application of XTEN technology has undergone evaluation in multiple classes of biotherapeutics including hormones, clotting factors and peptides as described earlier [19–21,25,56]. In addition, as the number of opportunities to utilize XTEN in chemical conjugation applications requiring half-life extension has matured, the family of XTEN polypeptides has expanded. Robust, scalable process platforms have emerged as a result of the respective, manifold product development efforts.

For both fusion products and the XTEN polypeptide stand-alone constructs employed for chemical conjugation, commonly used *E. coli* cell lines (K-12 and BL21 strains) have been adapted to serve as high-yielding expression platforms, with titers of 3–6 g/L reported [20,24,25]. XTENylated therapeutics have been expressed in mammalian cell culture systems as well (see Section 4.2).

Commonly employed plasmid backbones (e.g., pBR322, pACYC) used in the development of high-expression bacterial constructs, may be employed to enable reliable gene insertion. The incorporation of antibiotic resistance sequences (kanR or tetR) provides for efficient selection and control of product expression. Additional enhancements to the expression construct include insertion of the *cer* locus of the ColE1 plasmid, a binding site for the host encoded XerCD heterodimeric recombinase, which serves to resolve plasmid multimers and thereby improves plasmid stability. Conferral of enhanced plasmid stability by the *cer* locus has been demonstrated to be sufficient to enable antibiotic-free fermentation.

Because XTEN protein polymers have been engineered to provide very high solubility, the resulting fusion products are most conveniently expressed in the cytosol in soluble form. Depending on the genetic elements of the strain and expression vector employed, T7/lac or phoA promoter systems may be utilized to optimize product expression [101]. As desired, modifications to improve strain expression performance have been reported including selected deletions (e.g., ΔfhuA, ΔompT, ΔphoA) conferring added phage resistance, decreased proteolytic degradation and diminished levels of heat-shock protein overexpression, respectively.

Purification of soluble protein is aided by the enhanced solubility and thermodynamic stability typically exhibited by XTEN fusion products. By taking advantage of these beneficial attributes, robust clarification from fermentation matrices involving pH and heat treatment of product may be applied. The uniform, anionic topology and localized charge density imparted by the XTEN peptide portion of the expressed protein enables efficient, orthogonal chromatographic processing using conventional resins (e.g., both strong and weak cation and anion exchangers). By taking advantage of the extended anionic charge

localization (the pI of XTEN is ~3) to effect purification to homogeneity, consequent overall purification yields exceeding 30% are attainable [25].

Efforts to ensure enhanced stability and molecular integrity of the target protein attainable during expression, in some instances, require insertion of N- and C-termini modifications that include logically inserted proteolytic sites (e.g., tobacco etch virus protease and trypsin domains). Creating stable pre-protein expression constructs with built-in cleavage sites allows for attachment of His-tag domains and other stabilizing features [20,24,25] which can be used to further expand possible downstream processing options. Subsequent application of the appropriate enzymatic steps in the purification processing can be efficiently utilized to convert the pre-protein forms to the desired final product followed by quenching and removal of the unwanted enzymatic digestion by-products.

The adaption of XTEN technology to chemical conjugation techniques has enabled XTEN's application to the delivery of a broad spectrum of drug payloads [21,24]. The classes of compounds amenable for covalent linkage include a wide range of non-natural chemical moieties including D-isomers and other modified amino acid containing peptides or proteins as well as other synthetic molecules, to form modified chemical entities not feasible with genetic fusion. Chemical conjugation permits cross-linking of multiple copies of monospecific molecules to XTEN polymers in any chosen orientation, as well as of different drug classes to the same XTEN polypeptide via use of orthogonal chemistries. These conjugates may be produced through the precise positioning of chemically reactive amino acids on the XTEN backbone, resulting in molecular entities of unique composition, structure and activity. While typical bioactive molecules under investigation contain 1–3 linkages, up to nine chemical entities have been covalently conjugated to a single XTEN polypeptide, demonstrating the payload densities that can be carried by the XTEN backbone. Given XTEN's miscibility in organic solvents (e.g., acetonitrile, dimethylformamide and dimethyl sulfoxide) conventionally used in the generation of such covalent linkages, efficient and scalable coupling stoichiometries can be obtained. In addition, the allowance of organic phase processing serves to facilitate the subsequent application of final polishing purification steps, typically involving preparative scale reverse phase liquid chromatography.

6. Conclusions

XTENylation has been successfully tested as a viable and competitive half-life extension technology. Its broad adoption for the development of next-generation therapeutics will be significantly facilitated by the clinical success of lead candidates. Phase II clinical trials have been completed for XTENylated hGH (VRS-317); Phase III studies have been initiated in 2015 [69,70]. Multiple additional product candidates are in late preclinical stages.

XTEN protein polymers combine desirable physicochemical properties of synthetic polymers with the precision of DNA-encoded protein synthesis. The review has detailed both practical aspects of working with this class of polymers and current knowledge about the biological behavior of XTENylated molecules. The growing clinical experience and expanding knowledge base continue to drive discovery, enabling the creation of biopolymers with ever more precision-tailored functionality. It is foreseeable that XTEN can be used not only as a biodegradable bulking agent, but also as an assembly platform to combine multiple functionalities, such as targeting moieties and cytotoxic agents, into novel therapeutics for important clinical indications.

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